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Tumor microenvironment conditioning by abortive lytic replication of oncogenic γ-herpesviruses

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Abstract

Epstein Barr virus (EBV) and Kaposi sarcoma associated herpesvirus (KSHV) constitute the human γ-herpesviruses and two of the seven human tumor viruses. In addition to their viral oncogenes that primarily belong to the latent infection programs of these viruses, they encode proteins that condition the microenvironment. Many of these are early lytic gene products and are only expressed in a subset of infected cells of the tumor mass. In this chapter I will describe their function and the evidence that targeting them in addition to the latent oncogenes could be beneficial for the treatment of EBV and KSHV associated malignancies.

1. Introduction to human γ-herpesviruses

Among human herpesviruses, oncogenesis is confined to the γ-herpesviridae (1). These contain the lymphocryptovirus Epstein Barr virus (EBV) or human herpesvirus 4 (HHV4), and the rhadinovirus Kaposi sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8). Both viruses share a tropism for human B cells and stimulate these into lymphoproliferations in some of which even both viruses are present at the same time (2). In addition they are, however, also associated with tumors that originate from other cell types, including epithelial, NK/T and smooth muscle cells for EBV, and endothelial cell for KSHV (3, 4). The oncogenic potential of these two viruses is thought to originate from their need to differentiate B cells into long-lived memory compartments for persistence, memory B cells for EBV and plasma cells for KSHV (4, 5). These B cells get infected by the two viruses in sub-mucosal secondary lymphoid tissues after transmission via saliva exchange and possibly transcytosis across the mucosal epithelium (6). Latent infection by the two viruses is then thought to lead to B cell activation and proliferation. For EBV mainly eight latent gene products, more than forty miRNAs and two EBV encoded small RNAs (EBERs) are involved in this task (5). Differentiation from this activated B cell stage to memory B cells by follicular and extrafollicular routes allows for EBV persistence without any viral protein expression (7, 8). KSHV contains also three latent gene products which together with the viral miRNAs induce B cell proliferation after overexpression in mice (9). In addition, however, expression of some lytic KSHV gene products without overt infectious particle production augments B cell activation and differentiation, like the plasmablast or plasma cell differentiation that is induced by viral IL-6 (vIL-6) expression from the K2 locus (2). B cell proliferations that are driven by these viral programs can be found in human immunodeficiency virus (HIV) infected patients with acquired immunodeficiency syndrome (AIDS) as immunoblastic lymphoma that is associated with all EBV latent gene expression and multicentric Castleman's disease (MCD) that is associated with latent KSHV gene expression but also some lytic KSHV virus production (10, 11). These programs extensively shape the phenotype of the infected B cells to activated lymphoblasts by EBV and plasmablasts by KSHV, as well as modify their B cell receptor, introducing addition somatic hypermutation in the case of EBV, and switch to λ light chain expression in the case of KSHV (12, 13). From these stages both extrafollicular and germinal center dependent routes most likely lead to persistence with no viral protein and only viral non-translated RNA expression. At least EBV expresses a restricted set of latent proteins in centroblast and centrocytes to rescue infected B cells from cell death in germinal centers (14). From this reservoir both viruses reactivate into lytic infectious particle replication upon plasma cell differentiation for EBV and most likely also in plasma cells for KSHV (11, 15). If this occurs at

submucosal secondary lymphoid tissues, infectious virus can find its way into the saliva for further transmission, possibly after an additional amplification in mucosal epithelial cells at least for EBV (16). These lifecycles of EBV and KSHV utilize B cell immunobiology to both disseminate in their host, establish persistence in long-lived cells and allow reactivation in submucosal tissues for further transmission.

However, this lifestyle also forces them to induce B cell lymphoproliferations and channel infected cells through differentiation stages with increased somatic mutations. The oncogenic capacity of EBV and KSHV gene products and host gene mutations that emerge in the process of B cell differentiation lead to tumors associated with the two γ-herpesviruses. In this chapter I will discuss the different EBV and KSHV associated malignancies and how their microenvironments are conditioned for both pro-proliferative and immune evasive functions.

2. Classical oncogenes of EBV and KSHV

EBV is associated with tumors of B, epithelial, NK/T and smooth muscle cell origin (3). In these malignancies, EBV expresses a variable amount of latent viral proteins and the respective gene expression patterns are called latencies I, II and III. B cell derived immunoblastic lymphomas and post-transplant lymphoproliferative diseases (PTLDs) express all 8 latent EBV genes and are primarily observed during severe immune suppression, for example during advanced HIV infection and iatrogenic immune system inhibition (17). EBV associated smooth muscle tumors harbor also latency III (18). Latency II tumors like Hodgkin's lymphoma of B cell origin and nasopharyngeal carcinoma of epithelial cell origin express only one of the six nuclear antigens of EBV (EBNAs), namely EBNA1, and the two latent membrane proteins LMP1 and 2 (1, 19). This latency II or even less latent viral protein expression can also be found in NK/T cell lymphomas that are associated with EBV (20). Finally, only EBNA1 is expressed in latency I which is found in Burkitt's lymphoma and primary effusion lymphoma (PEL), the latter being in the majority of cases also co-infected with KSHV (1). In contrast to these distinct latent EBV gene expression patterns, KSHV rarely expresses only its three latent viral proteins, latency-associated nuclear antigen (LANA), viral FLICE-like inhibitory protein (vFLIP) and viral cyclin (vCYC), and viral miRNAs (4). The adjacent kaposin locus K12 is also often expressed, as well as in decreasing frequency the K15, the K2 and the non-translated polyadenylated nuclear RNA (PAN) encoding KSHV genome region (21, 22). This variable gene expression is seen in both endothelial and B cell derived tumors that are associated with KSHV, namely Kaposi sarcoma or multicentric Castleman's disease (MCD) and PEL (23-25). It might represent a variable frequency of cells undergoing abortive and productive lytic KSHV replication in the respective tumors (26). Thus, variable viral gene expression patterns can be observed in γ-herpesvirus associated malignancies. These segregate with tumor entities for EBV and subdivide tumor entities for KSHV.

Both viruses contain bona fide oncogenes, which upon expression in mice cause tumors. This has been shown for the viral latency locus and vFLIP of KSHV (9, 27, 28), and for EBNA1 and LMP1 of EBV (29, 30). Interestingly, both viruses activate c-myc and NF-κB to induce B cell activation and proliferation. EBV achieves this via EBNA2 assisted c-myc transcription (31) and LMP1 mediated constitutive NF-κB activation (32). In some EBV associated lymphomas that express only EBNA1 the c-myc expression is achieved by cellular mutations that are thought to compensate for EBNA2 absence, like c-myc translocation into the immunoglobulin loci for Burkitt's lymphoma (33) and c-myc gene amplification in lymphomas that emerge in mice upon EBNA1 expression in B cells (34). For KSHV, LANA amplifies c-myc activity (35, 36) and vFLIP activates NF-κB (37, 38). These pro-proliferative functions are paired with anti-apoptotic mechanisms, such

as for EBV the EBNA3C mediated inhibition of pro-apoptotic $p16^{INK4a}$ and BIM expression (39, 40) and the pro-survival B cell receptor like signaling of LMP2 (41). In PEL the p16INK4a locus is sometimes mutated to presumably compensate for the absence of an active mechanism to suppress this pro-apoptotic protein, which is induced by the cell cycle driving activity of vCYC (42). One can also speculate that the B cell receptor modifying activities, somatic hypermutation by EBNA3C mediated activation induced deaminase (AID) induction (12) and λ chain usage driven by vFLIP (28), might improve tonic signaling for infected B cell survival, similarly to LMP2 function. In addition to these immunoblastic features of latent EBV and KSHV infection, the leaky lytic KSHV gene product expression, mainly from the K2 locus encoding vIL-6 (Figure 1) induces plasma cell features in MCD and PEL (43, 44). However, the functions of latent γ-herpesvirus proteins and leaky presumably abortive lytic gene expression go much further than just transforming EBV and KSHV infected cells. They also heavily condition the microenvironment of the associated tumors and this regulation will be discussed next.

3. Conditioning of the tumor microenvironment by lytic and latent EBV and KSHV gene expression

During their co-evolution with the human host both EBV and KSHV have reached a stalemate with the immune system that in the vast majority of the more than 90% of adults that are persistently infected with EBV and of the more than 75% of adults that have encountered KSHV in some sub-Saharan countries leads to persistence of both viruses, but also does not cause pathology (45, 46). On the contrary KSHV and EBV seem to even promote this equilibrium. For example, EBV encodes with EBNA3B a tumor suppressor (47). This latent viral nuclear antigen induces transcription of CXCL9 and 10, two inflammatory chemokines that recruit lymphocytes via their CXCR3 receptor. EBV deficient in EBNA3B causes lymphomas at increased frequencies with diminished inflammatory infiltrates, and restoration of CXCL10 secretion by transfection also reinstalls T cell mediated immune control of lymphoma cells with EBNA3B deficient EBV. Not only latent gene products, but also early lytic EBV proteins foster leucocyte recruitment to the vicinity of infected cells (Figure 1). Along these lines CCL5 production has been reported in lymphomas with higher lytic EBV replication (48). This chemokine facilitates macrophage recruitment via CCR5 binding into the tumor microenvironment (49). Similarly, KSHV encodes three macrophage inflammatory protein (MIP) homologues (vCCL1-3) as early lytic gene products. These are thought to recruit myeloid cells into the microenvironment of KSHV infected cells via CCR8 (vCCL1 and 2) and XCR1 (vCCL3) (50). These inflammatory infiltrates protect persistently EBV and KSHV infected hosts probably most of the time from γ-herpesvirus associated pathologies.

However, in virus associated tumors these infiltrates are turned into tumor cell nurturing and immunosuppressive leucocytes. Along these lines EBV encodes viral IL-10 (51, 52) and KSHV associated lymphomas are also dominated by IL-10 production (53). IL-10 suppresses T cell mediated restriction of EBV transformed B cells (54, 55) (Figure 1). In addition, tumor cells as well as inflammatory infiltrates produce TGF-β in Hodgkin's lymphoma (56), which is presumably involved in the induction of regulatory T cells rosetting around the malignant Reed-Sternberg cells in this tumor entity (57). Furthermore, EBV encoded viral miRNAs also compromise MHC class I restricted antigen presentation to $CD8^+$ T cells and their CXCL11 mediated attraction into the tumor microenvironment (58-61), further dampening T cell mediated immune control (Figure 1). While EBV miRNAs compromise MHC restricted antigen presentation both during latency and lytic replication, early lytic gene products of both viruses further compromise MHC class I restricted $CD8^+$ T cell stimulation. These are the KSHV K3 and K5 gene products that down-regulate MHC class I molecules (62) and the EBV BGLF5, BNLF2a and BILF1 gene products that inhibit MHC class I transcription, peptide loading and surface expression, respectively (63). Both

viruses contain with KSHV vIRF1-4 and EBV BZLF1, BRLF1 and BGLF4 also lytic gene products that block interferon signaling (46, 63), but at least for EBV there is little evidence that type I IFN influences its infection in vivo (64). Thus, lytic gene expression of γ-herpesviruses in a subset of cells in the associated tumors and often not resulting in productive replication of infectious particles, conditions the respective tumor microenvironment to be immune suppressive and amplifies this immune suppression by infiltrating leucocyte polarization.

In addition to immune modulation in the tumor microenvironment, lytic KSHV gene products also contributes to angiogenesis and thereby further supports tumor growth. KSHV G protein-coupled receptor (vGPCR), K1 and K15 stimulate angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and angiopoietin 2 (ANGPT2) (26, 46) (Figure 1). This pro-angiogenic functions of KSHV might be an adaptation to the viral life cycle in endothelial cells, giving rise to Kaposi sarcoma, even so it remains unclear why KSHV requires endothelial cell infection for persistence.

4. Targeting lytic replication for the treatment of EBV and KSHV associated malignancies

Even so lytic viral replication induction should intuitively destroy tumor cells, the above discussed contributions of early lytic gene products to paracrine microenvironment conditioning for optimal tumor growth might make it attractive to inhibit lytic γ-herpesvirus infection as a treatment for EBV and KSHV associated malignancies. Along these lines EBV deficient in lytic infection induction causes less tumors in mice with from $CD34^+$ hematopoietic progenitor cells reconstituted human immune system compartments (humanized mice) (65, 66). Furthermore, EBV strains with increased lytic replication are enriched in malignancies that are associated with this virus (67-69). Similarly in KSHV associated MCD, inhibition of the lytic cycle associated viral DNA polymerase with a combination of zidovudine and valganciclovir was clinically efficacious in the majority of cases (70). Furthermore, in HIV infected individuals that were treated with the herpesviral DNA polymerase inhibitor ganciclovir for human cytomegalovirus (HCMV) reactivation Kaposi sarcoma incidence was significantly reduced (71, 72). Finally, herpesviral DNA polymerase inhibition has also been successful in individual cases of PELs (73). These studies indicate that lytic EBV and KSHV replication might enhance virus associated tumorigenesis and should be targeted for treatment.

However, instead of inhibition of overall lytic γ-herpesviral infection, individual effects of lytic EBV and KSHV gene expression can also be targeted. Along these lines CCR5 that has been suggested to mediate recruitment of myeloid cell into the tumor microenvironment of Hodgkin's lymphoma has been inhibited with maraviroc in combination with blocking antibodies against its ligand CCL5 (74). CCL5 is thought to be elicited by early lytic EBV infection (48). Blocking CCL5 binding to CCR5 inhibited Hodgkin's lymphoma growth in a xenograft model. Similarly, VEGF that is induced by KHSV vGPCR, K1 and K15 has been blocked with the recombinant antibody bevacizumab in Kaposi sarcoma patients (75). This led to a clinical response in around 30% of treated individuals. Furthermore, the early lytic KSHV gene product vIL-6 is thought to drive plasma cell differentiation in MCD and PEL (2). Plasma cell differentiation renders tumors susceptible to proteasome inhibition, as seen for multiple myeloma (76). Indeed, combining the proteasome inhibitor bortezomib with chemotherapy successfully treated PEL in one patient (77). IL-6 receptor was also directly targeted for treatment of MCD with clinical efficacy in a few patients (78, 79). These initial encouraging results suggest that also individual lytic γ-herpesvirus gene products and their effects can be inhibited for therapeutic benefit.

These individual lytic EBV and KSHV proteins can also be used as active or passive vaccine antigens to target the above discussed paracrine functions. Along these lines the protective value of late lytic EBV antigen specific $CD4^+$ T cell responses have been explored in a

humanized mouse model (80). The respective viral antigens also sensitized neighboring latently infected cells for $CD4^+$ T cell recognition after transfer from the subset of lytically EBV replicating cells. However, in active vaccination with EBV derived viral particles addition of the latent EBNA1 antigen improved protective vaccine efficacy (81). Nevertheless, lytic EBV antigens should be considered in combination with latent antigens for an optimal vaccine formulation to elicit protective T cell responses.

5. Conclusions and future outlook

Human γ-herpesviruses contain some of the most oncogenic pathogens. Apart from their oncogenes, some of the EBV and KSHV associated malignancies, however, heavily rely also on their inflammatory infiltrates to sustain tumor growth. This is probably most dramatic in Hodgkin's lymphoma in which only around 1% of the tumor mass represents the malignant Reed-Sternberg cells (57). It has become apparent in the recent years that paracrine conditioning of this tumor microenvironment by a small subset of cells undergoing lytic γ-herpesvirus infection serves functions in the recruitment of immune cells, immune suppression and angiogenesis. We now need to capitalize on these findings for new treatments of EBV and KSHV associated malignancies that are more specific for these viruses than B cell depletion and overall inhibition of herpesviral DNA polymerases.

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Figure legend

Figure 1: Conditioning of the tumor microenvironment of EBV and KSHV associated malignancies. The microenvironment of both EBV (left) and KSHV (right) associated malignancies is composed of a mixture of latently and early lytically (BZLF1 or Rta) infected cells. Early lytic replication conditions the microenvironment of both EBV and KSHV associated malignancies by attracting monocytes to differentiate into immune suppressive tumor associated macrophages (TAM) via CCL5 or viral macrophage inflammatory proteins (vMIP). Furthermore, viral IL-10 (vIL-10) suppresses immune activation in the microenvironment of EBV associated malignancies and viral IL-6 (vIL-6) induces plasma cell differentiation in KSHV associated malignancies. EBV further suppresses $CD8^+$ T cell mediated immune control by blocking CXCL11 mediated attraction of $CD8^+$ T cells and down-regulation of MHC class I restricted antigen presentation with its miRNAs that are expressed during latency and lytic infection. Early lytic KSHV infection is pro-angiogenic, triggering vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and angiopoietin 2 (ANGPT2) production.

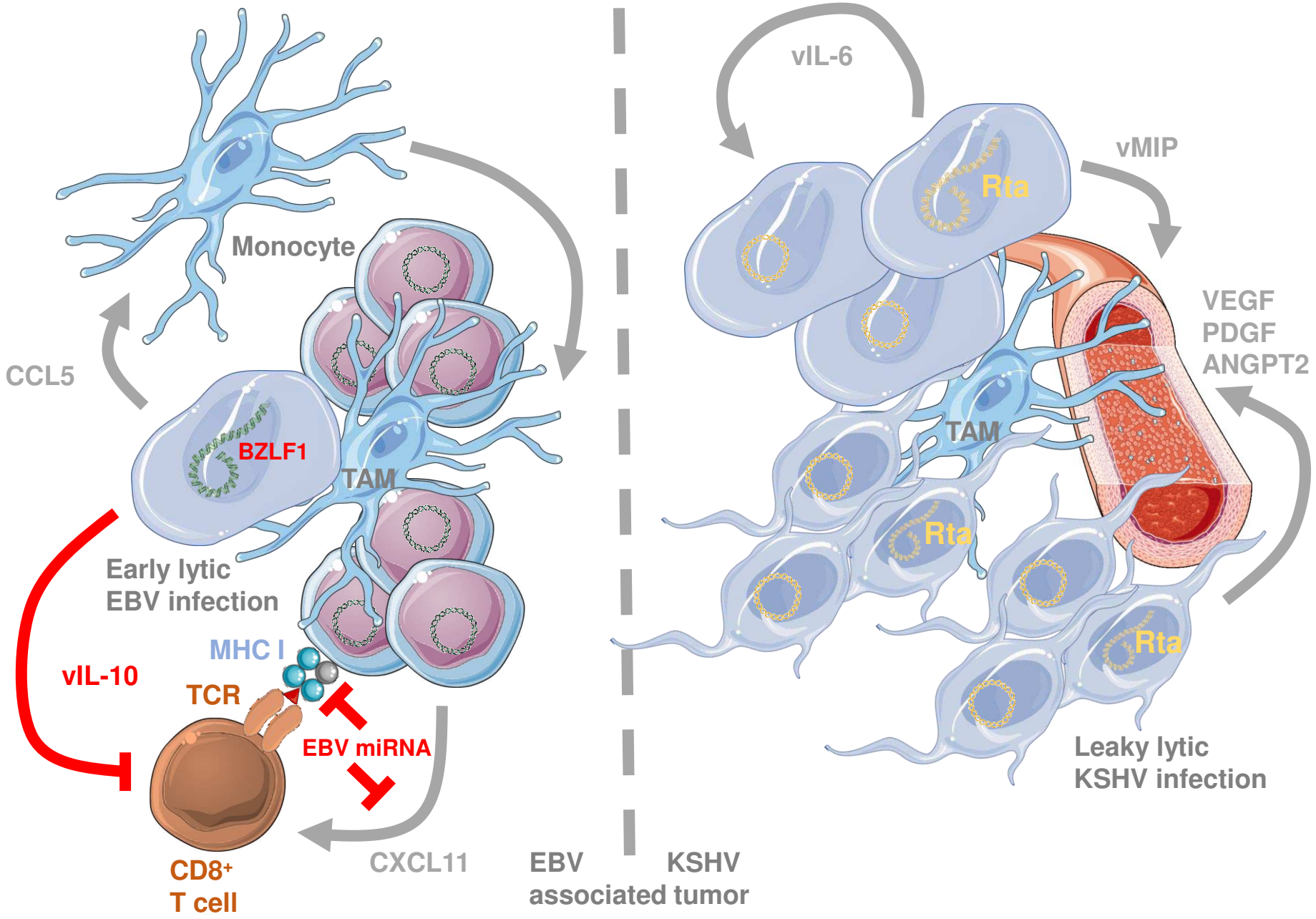
Monocyte
CCL5
BZLF1
TAM
Early lytic
EBV infection
MHC I
vIL-10
TCR
EBV miRNA
CD8+
T cell
CXCL11
EBV
associated tumor
KSHV
vIL-6
vMIP
Rta
VEGF
PDGF
ANGPT2
TAM
Rta
Rta
Leaky lytic
KSHV infection

Figure 1